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A Novel Assay for Hepatitis B e Markers

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The evaluation of a novel assay that allows simultaneous testing for hepatitis B e antigen and its antibody in a single well is described. The results of routine application and sequential studies on patients with acute hepatitis B and chronic hepatitis B treated with interferon are presented. The specificity and sensitivity of the assay and its ability to be used to follow the response of a patient during the whole seroconversion episode has been evaluated. The assay proved to give useful information about the reactivity of the sample, especially in those patients who were changing their ''e'' status. ***J. Med. Virol. 52:280–285, 1997.***

KEY WORDS: hepatitis B e antigen assay; HBeAg seroconversion; simultaneous assay

INTRODUCTION

The hepatitis B e antigen (HBeAg) and its antibody (anti-HBe) were first described by Magnius and Espmark [1972] using simple double immunodiffusion techniques. Since that time, the presence of HBeAg has been shown to correlate with the presence of viral particles and hepatitis B virus DNA (HBV DNA) in serum. The presence of HBeAg in serum correlates with increased infectivity in both inoculation accident [Alter et al., 1976] and perinatal transmission settings [Stevens et al., 1975]. Sequential assay of both HBeAg and anti-HBe in both acute and chronic hepatitis patients gives additional information on the stage of the infection and the infectivity of the patient. This is required to formulate advice with regard to prophylaxis for contacts (e.g., in health care settings) and for the babies of hepatitis B carrier mothers. Further, the widespread use of interferon therapy has brought with it a requirement for the laboratory to provide as much information as possible about the response of the patients in order to optimise therapy.

Conventional immunoassay systems for HBeAg and anti-HBe use common reagents but require separate assays to be carried out for each marker. The HBeAg assay is usually a direct binding assay in which a high signal (radioactive count or optical density) indicates a positive sample reactivity. Using the same reagents, anti-HBe is usually measured using a competition assay in which measured amounts of HBeAg (sometimes called neutralising agent) is added to the assay and anti-HBe in the patient's sample blocks the labelled or conjugated antibody, giving a low signal. Results are evaluated with respect to calculations carried out on negative control samples. Using such assay systems, the cutoffs of the assays can be numerically quite close to each other. HBeAg-positive samples having values above the cutoff and anti-HBe-positive samples giving values below the cutoff. Using these systems, a sample could be (1) positive for HBeAg, (2) positive for anti-HBe, (3) negative for both HBe and anti-HBe, or (4) positive for both HBeAg and anti-HBe.

The progression from HBeAg positivity to anti-HBe positivity is one that occurs naturally in about 2–15% carriers per year [Lever, 1988; Viola et al., 1986; Fattovitch et al., 1986]. Such seroconversions are often accompanied by a clinical or subclinical episode of "hepatitis," although the mechanisms initiating these changes are not fully understood.

As HBeAg is a soluble protein produced during viral replication, its presence in serum permits take-up by the immune system and consequent production of anti-HBe. In each patient, there must be a unique balance between the production of both markers; immunoassay systems permit detection of whichever is in excess. If viral replication stops and HBeAg is no longer produced, anti-HBe will complex and will slowly eliminate all HBeAg from the circulation. Anti-HBe will then predominate and gradually increase in titre. The assay described in this paper allows a numerical assessment of this dynamic process. By combining the assay for HBeAg and anti-HBe in one simultaneous assay, the evaluation of reactivity is assessed from the position on a single continuum of results, ranging from high levels of HBeAg to high levels of anti-HBe. Similar assays

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have been described for hepatitis B surface antigen (HBsAg) and antibody (Immuno Ltd., HBsAg/Anti-HBs RIA quick pack leaflet. Ref. 7260000E201/79 g, h, i) [Atherton and Boxall, 1986] and HBeAg and anti-HBe [Ferns and Tedder, 1985], using a radioactive probe that can give a wide dynamic range of assay values. The use of enhanced chemiluminescent technology gives a wide range of quantitative values using a non-radioactive system.

Hepatitis B e antigen has been shown to have two immunodominant epitopes designated α and β and are both recognized by each patient when an antibody response is mounted [Matsuyama et al., 1985]. The presence of two epitopes has been exploited in the combined HBeAg and anti-HBe assay and this principle has been extended using a preformed complex described below for the Amerlite assay.

METHODS

Hepatitis B markers were assayed using commercial immunoassays as follows: HBsAg by enzyme immunoassay, (EIA Bio Products Laboratories and Organon Uniform II); HBsAg was titrated using reverse passive haemagglutination (Hepatest; Murex); anti-HBs by enhanced luminescent immunoassay (ELIA; Amerlite, Ortho Clinical Diagnostics), anti-HBc IgM by ELIA, (Amerlite; Ortho Clinical Diagnostics); and HBe and anti-HBe by a bead-based radioimmunoassay (RIA) (Abbott Laboratories), as well as the Ortho Amerlite HBe/anti-HBe assay. Assay results are expressed as test results divided by cutoff, except for HBsAg titre, which is expressed as reciprocal titre. HBV-DNA assays were by solution hybridisation using an I-125-labelled probe (Genostics, Abbot Laboratories).

HBsAg-positive samples were from patients detected through screening programmes or from patients under investigation for acute or chronic liver disease or who were being treated as indicated. HBsAg-negative samples were from patients with recent viral infection, including hepatitis A, from patients with renal failure or nonviral liver disease, and from HBsAg-negative blood donors. For the serial sample, studies archived samples collected over an extensive period were studied.

Amerlite HBe/Anti-HBe Assay

In the Amerlite HBe/Anti-HBe assay, the wells are coated with mouse monoclonal antibody to HBeAg of α specificity and the conjugate contains mouse monoclonal antibody to HBeAg of β specificity. The conjugate is precomplexed with a small amount of HBeAg. During the single incubation step, patient sample or control and the enzyme conjugate anti-HBe are mixed in each microwell. If neither anti-HBe or HBeAg is present in the sample (e.g., for an HBV-negative sample), a small quantity of the complex binds to the anti-HBe-coated well via the HBeAg component, resulting in a moderate signal. This signal therefore corresponds to a nonreactive result for the negative control or for a negative sample. In the case of an HBeAg-positive sample, additional conjugate is bound via the additional sample HBeAg to the well antibody resulting in a signal elevated above that of the negative control. With an anti-HBe positive sample, the sample antibody binds to the HBeAg in the complex through the α epitopes and the β epitopes, remaining uncomplexed with the added antigen. Binding of the enzyme-immune complex to the anti-α antibody on the well is effectively shielded and the signal is reduced below that of the negative control (Fig. 1).

When HBeAg and anti-HBe are present at equivalence, the result will be similar to that of HBV-negative sample, although other hepatitis markers will be positive. This has been described for HBe/anti-HBe, where up to 6% of HBsAg-positive samples were negative for HBeAg as well as for anti-HBe [Matsuyama et al., 1985].

Following incubation, unbound material is removed by washing of the wells, followed by the addition of the Amerlite chemiluminescent reagent. The light signals are read in the Amerlite analyzer. For light signals above those of the negative control, the amount of conjugate bound is directly related to the level of HBeAg detected; for light levels below those of the negative control, the amount of conjugate bound is inversely related to the level of anti-HBe detected.

Results are calculated and expressed in this paper as a normalized signal relative to the kit negative control signal. A result of 0.0–0.45 indicates that detection of anti-HBe antibody, 0.45–1.3 is nonreactive and indicates that neither anti-HBe nor HBeAg have been detected. A nonreactive sample contains (1) neither HBeAg nor anti-HBe at detectable levels, or (2) both HBeAg and anti-HBe in equivalence. An assay result of >1.3 indicates detection of HBeAg in the sample. The cutoff values of 0.45 and 1.3 were established after extensive "in-house" studies and external field trials, using clinical samples.

RESULTS

Assays for HBe markers by the Amerlite assay and Abbott Laboratories HBeAg/anti HBe RIA were carried out on a range of samples. In addition, quality control sera for HBeAg and anti-HBe were included in assays as appropriate. Five quality-control samples were tested in each assay to assess within-assay and between-assay precision. These controls included both a strong and a weak HBeAg, along with a known anti-HBe, a HBsAg-negative sample, and a sample containing a mixture of HBeAg and anti-HBe.

This assay was performed for 174 patient sera, 54 from patients negative for HBsAg and 120 from HBsAg-positive patients, 40 HBeAg positive, 60 anti-HBe positive, and 20 negative for both e markers by the Abbott RIA. Among the samples negative for hepatitis B markers were 8 samples positive for hepatitis A IgM antibodies, 5 from patients with other recent viral infections, 15 patients with chronic renal failure, and 12 with nonviral liver disease. Two of the HBsAg-negative samples were positive for anti-HBe, both from patients who were naturally immune to hepatitis B and also

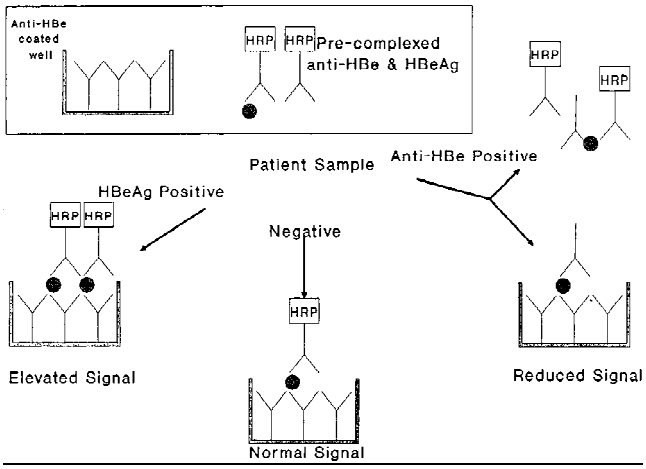

Fig. 1. Principle of the Amerlite HBe/anti HBe assay. Opaque microtitre wells are coated with anti-HBe. Conjugate complex contains monoclonal anti-HBe plus precomplexed HBe antigen. If no sample is added, or if the sample is negative for HBe antigen and anti-HBe, a signal is still produced. If the sample contains HBe antigen, an elevated signal is obtained. If the sample contains anti-HBe, a reduced signal is produced.

anti-HBe positive by RIA; all other HBsAg-negative samples were nonreactive for HBeAg and anti-HBe.

Table I shows the results of the Amerlite and Abbott RIA HBe assays on the HBsAg-positive samples.

HBeAg positive. Thirty-eight of 40 samples classified as HBeAg positive by the Abbott assay were also HBeAg positive by the Amerlite assay. The two other samples were classified by the Amerlite assay as repeatably nonreactive for both HBe and anti-HBe. Both samples were repeatably reactive for HBeAg by the Abbott assay, neither strongly so, with both within 40% of the cutoff level. There were no false-positive results, and no HBeAg samples were assigned anti-HBe reactivity. The sensitivity of the Amerlite assay with respect to RIA for the detection of HBeAg is therefore 95%; specificity is 100%, as is positive predictive value.

Anti-HBe positive. All 60 samples found to be anti-HBe positive by Abbott RIA were anti-HBe positive by the Amerlite assay. There were no false-positive results, and no anti-HBe samples were assigned HBeAg positivity. Sensitivity, specificity, and positive predictive value are 100%.

HBeAg/anti-HBe negative. Twenty samples assigned this category by the Abbott assay all proved nonreactive in the Amerlite HBe/anti-HBe assay. Two samples very close to the cutoff value for anti-HBe in the Amerlite assay were also close to the cutoff for anti-e in the Abbott RIA.

Reproducibility

Within-assay precision was measured over seven measurements of samples set up in duplicate on one plate. The coefficient of variation was less than 10% for a strong HBeAg positive, less than 7% for a weak HBeAg, and 7.0–10.6% for nonreactive samples. The coefficient of variation between assays was also less than 10%, when measured for seven assays. Variation between different kit lots was not investigated.

TABLE 1. Comparison of HBeAg/Anti-HBe: Results of Patient Samples

	Abbott RIA			
Amerlite	HBeAg	Nonreactive	Anti-HBe	Total
HBeAg	38	0	0	38
Nonreactive	2	20	0	22
Anti-HBe	0	0	60	60
Total	40	20	60	120

HBeAg, hepatitis B e antigen; RIA, radioimmunoassay.

Serial Dilution Studies

An HBeAg-positive and an anti-HBe-positive sample were diluted in a serum negative for hepatitis B markers; the results are shown in Figure 2. Abbott RIA and Amerlite were close to equivalent sensitivity for both HBeAg and anti-HBe.

Serial Samples

The results of serial assay on patients with acute hepatitis B and patients on interferon therapy are shown—(acute: Tables II and III; chronic; Tables IV–IX.

Acute hepatitis. The two patients described here were detected on routine screening while still in the incubation phase of their infection, with A1 representing a blood donor and A2 a patient attending a clinic for sexually transmitted diseases. Both patients were initially negative for HBe antigen but became positive in one case before, and in the other case at the same time as the appearance of anti-HBc. Patient A1 is unusual in that no samples were shown to be reactive for IgM class antibody to HBcAg; however, this patient was a regular blood donor who had been HBsAg negative 6 months previously, but had recently married a lady from an area endemic for hepatitis B. We have recently noted that some patients with an acute hepatitis B infection can eliminate anti-HBc IgM very rapidly (N.S. Khaihrulla and E.H. Boxall, in preparation), and the timing of samples from this patient may be such that its expression was missed. In such cases the diagnostic significance of changes in HBe/anti-HBe is heightened.

In our studies, patients have presented with acute hepatitis B when either already HBeAg positive or nonreactive for "e" markers, while strongly positive for HBsAg and anti-HBc IgM. Most of these patients were found to be anti-HBe positive at follow-up evaluation.

Patients on interferon therapy. The results of serial assays on six chronic carriers of hepatitis B undergoing interferon therapy are shown in Tables IV–IX. Patients were treated with recombinant interferon-α (IFN-α) for a period of 16 weeks, some following pretreatment with steroids. Tables IV–IX indicate the period of treatment (IFN). Patients C1–C3 were successfully treated with IFN, eliminating both HBeAg, HBsAg, and HBV DNA.

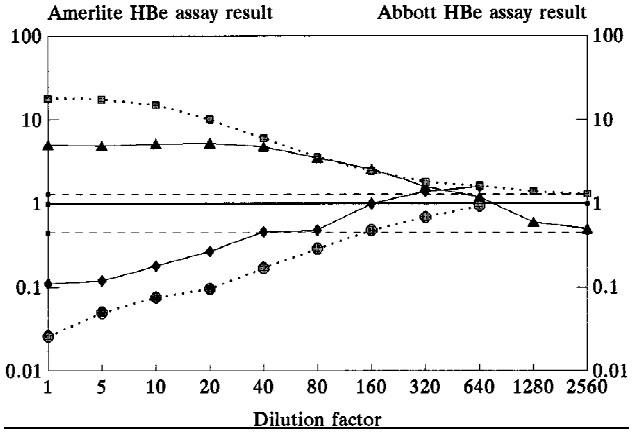

Fig. 2. Assay results by Amerlite and Abbott RIA on HBeAg and anti-HBe-positive samples diluted serially. - - - ■ - - -, Amerlite result (HBe Ag); - - - ⬣ - - -, Amerlite result (anti-HBe); —▲—, Abbott result (HBeAg); — ◆ —, Abbott result (anti-HBe). Dashed lines show Amerlite cutoffs. Abbott cutoff for both tests is 1.0.

TABLE II. Acute HBV Serial Samples: Results of Marker Assays—Patient A1

Time (wk)	HBsAg titre	Anti-HBc total/IgM	Amerlite e assay	Interpretation
0	+RIA	I/S	0.82	—
6	1:8,000	–/–	15.5	HBeAg
11	1:4,000	+/–	5.1	HBeAg
13	1:256	+/–	0.59	—
15	<1:4	+/–	0.6	—
17	<1:4	+/–	0.37	Anti-HBeAg
21	Negative	+/–	0.41	Anti-HBeAg

HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; RIA, radioimmunoassay. Anti-HBc, antibody to hepatitis B core antigen total and immunoglobulin M class.

TABLE III. Acute HBV Serial Samples: Results of Marker Assays—Patient A2

Time (wk)	HBsAg titre	Anti-HBc total/IgM	Amerlite e assay result	Interpretation
0	+<1:4	–/–	0.83	—
1	+<1:4	–/–	1.02	—
5	+<1:4	+/+	5.4	HBeAg
8	1:4,000	+/+	0.15	Anti-HBe
10	1:1,000	+/+	0.067	Anti-HBe
11	1:512	+/+	0.008	Anti-HBe
14	<1:4	+/+	0.021	Anti-HBe
15	Negative	+/+	0.064	Anti-HBe

HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBe, hepatitis B e. Anti-HBc, antibody to hepatitis B core antigen total and immunoglobulin M class.

Patient C1 showed a fall in the HBeAg assay value soon after starting interferon. The value continued to fall, becoming weakly anti-HBe positive before becoming more strongly anti-HBe reactive on follow-up. Patient C2 had a lower starting level of HBeAg and, without going through a nonreactive phase, became weakly reactive for anti-HBe, but on follow-up became nonreactive. This patient became anti-HBe positive by Amerlite while still weakly reactive for HBeAg by RIA; the same situation was observed with patient C5.

TABLE IV. Chronic HBV on Interferon Treatment: Results of Marker Assays—Patient C1

Time (wk)	HBsAg titre	HBV DNA (pg/ml)	Amerlite e assay result	Interpretation
0	>8,000	72	14	HBeAg
8 IFN	>8,000	—	11.2	HBeAg
12 IFN	1:256	0	7.1	HBeAg
16 IFN	<1:4	—	0.39	Anti-HBe[a]
25 IFN	—	0	0.43	Anti-HBe[a]
29 IFN	—	0	0.59	—
34	—	0	0.78	—
74	—	0	0.18	Anti-HBe

[a]Within 10% of cutoff.
HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IFN, interferon.

TABLE V. Chronic HBV on Interferon Treatment: Results of Marker Assays—Patient C2

Time (wk)	HBsAg titre	HBV DNA (pg/ml)	Amerlite e assay result	Interpretation
0	1:8,000	190	7.1	HBeAg
2 IFN	>1:8,000	102	6.9	HBeAg
6 IFN	1:512	—	0.29[a]	Anti-HBe
11 IFN	1:8	—	0.28	Anti-HBe
16 IFN	<1:4	0	0.33	Anti-HBe
45	Negative	0	0.51	—

[a]This sample weakly reactive for HBeAg by RIA.
HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IFN, interferon.

TABLE VI. Chronic HBV on Interferon Treatment: Results of Marker Assays—Patient C3

Time (wk)	HBsAg titre	HBV DNA (pg/ml)	Amerlite assay result	Interpretation
0	1:4,000	6	16.2	HBeAg
3	1:8,000	5	16.9	HBeAg
7 IFN	1:4,000	—	16.7	HBeAg
11 IFN	1:8,000	—	17.1	HBeAg
15 IFN	1:2,000	—	14.4	HBeAg
23 IFN	+RIA	—	0.77	—
27	—	0	0.92	—
42	—	—	0.85	—

HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IFN, interferon.

Patient C3 successfully cleared HBsAg and HBeAg without becoming reactive for anti-HBe, although samples were taken at approximately monthly intervals for almost 1 year.

Patient C4 showed a seroconversion to anti-HBe going through a nonreactive phase by Amerlite. Samples from the start and end of the nonreactive phase proved reactive for both HBe and anti-e by RIA. The last two samples from this patient indicated an increasing anti-HBe titre.

Patient C5 seroconverted to anti-HBe within a few weeks of starting interferon, went on to become more strongly positive for anti-HBe (value 0.003), but remains HBV DNA positive. The earliest sample showing

TABLE VII. Chronic HBV on Interferon Treatment: Results of Marker Assays—Patient C4

Time (wk)	HBsAg titre	HBV DNA (pg/ml)	Amerlite e assay result	Interpretation
0	1:8,000	9	14.2	HBeAg
21 IFN	1:8,000	14	10.0	HBeAg
22 IFN	1:8,000	—	1.86	HBeAg
25 IFN	1:8,000	0	0.6[a]	—
27 IFN	1:2,000	0	0.57	—
31 IFN	1:8,000	0	0.5[a]	—
39 IFN	1:8,000	0	0.32	Anti-HBe
52	1:4,000	0	0.024	Anti-HBe

[a]These samples were reactive both for HBeAg and anti-HBe by RIA.
HBsAg, hepatitis B surface antigen; HBeAg, hepatitis Be antigen; HBV, hepatitis B virus; IFN, interferon.

TABLE VIII. Chronic HBV on Interferon Treatment: Results of Marker Assays—Patient C5

Time (wk)	HBsAg titre	HBV DNA (pg/ml)	Amerlite e assay result	Interpretation
0	1:4,000	100	NT	I/S[b]
32 IFN	1:8,000	11	11.1	HBeAg
36 IFN	1:512	0	0.2[a]	Anti-HBe
42 IFN	1:512	NT	0.062	Anti-HBe
43 IFN	+RIA	4.2	0.003	Anti-HBe
52 IFN	+RIA	3.2	0.003	Anti-HBe
59	1:128	16	0.003	Anti-HBe
66	+RIA	25	0.059	Anti-HBe
91	+1:256	23	0.003	Anti-HBe

[a]HBeAg positive by RIA.
[b]Insufficient sample.
HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IFN, interferon; RIA, radioimmunoassay. NT, not tested.

TABLE IX. Chronic HBV on Interferon Treatment: Results of Marker Assays—Patient C6[a]

Time (wk)	HBsAg titre	HBV DNA (pg/ml)	Amerlite e assay result	Interpretation
0	1:1,000	105	17.2	HBeAg
51	1:1,000	123	11.0	HBeAg
86	1:2,000	13.0	14.8	HBeAg
91	RIA	2.5	18.1	HBeAg
100	1:32	6.8	10.9	HBeAg
108	1:32	8.1	9.1	HBeAg
115	1:256	9.3	11.3	HBeAg

[a]Interferon started on week 64 ended week 80.
HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; RIA, radioimmunoassay.

seroconversion to anti-HBe was still positive for HBeAg, as determined by RIA.

Patient C6 is the sibling of C5. The results show no change in HBe status with treatment. Although there is a fall in HBV DNA level, the patient did not produce a lasting response to antiviral therapy with interferon.

DISCUSSION

Sensitivity Specificity and "Added Value" of Simultaneous Assay

In routine use, the Amerlite assay was shown to be of equivalent sensitivity and specificity as the Abbott RIA for anti-HBe, but for HBeAg the sensitivity was slightly reduced. However, the two samples missed by the Amerlite assay were only weakly reactive for HBeAg by RIA. Serial dilution of an HBeAg reactive sample and an anti-HBe reactive sample showed the two assay systems to be of approximately equivalent sensitivity.

In developing a simultaneous assay, it is likely that some minor compromise in sensitivity will be sacrificed. In return, more information is given about the reactivity of the sample. By allocating all HBsAg-positive sera onto a single numerical scale ranging from 0.001 (strong anti-HBe) through nonreactive (0.45–1.3) to about 20 units (strong HBeAg), judgments can be made about the degree of reactivity, which can aid the interpretation of the results beyond simply assigning HBeAg or anti-HBe positivity. Very sensitive assays detecting very low levels, particularly of HBeAg, may cause concern over the specificity of weak reactions and how such reactivities should be interpreted. The wide dynamic range of the enhanced luminescent assay gives sufficient flexibility for a simultaneous test to work well in practice and the numerical value generated by the system has added value in interpretation.

The progression from HBeAg reactivity to anti-HBe reactivity is a dynamic process that is unique to each patient, the nonreactive phase by Amerlite may be reactive for both markers by assays with enhanced sensitivity, but the interpretation may not be different. The interpretation of the results of assays on single sera can be difficult. It is not uncommon in diagnostic virology to base clinical interpretation on the results of more than one serum. The interpretation of e markers should be no different, as amply demonstrated by the serial studies on patients with both acute and chronic HBV infections described here.

Serial Samples

Tables II–IX illustrate the range of individual responses to infection and therapy. A range of HBeAg assay values at presentation and resolution were observed. Loss of HBV DNA and HBeAg was normally associated with an anti-HBe response (see patients C1, C2, C4, C5), but not in every patient (C3). Not all HBeAg chronic carriers respond to interferon treatment. For example, patient C6 retained HBsAg, HBV DNA, and HBeAg despite therapy, and the Amerlite HBe assay result remained consistently high over a period of 2 years. A fall in HBeAg value is therefore a more reliable indicator of lasting response to interferon than a fall in HBV DNA while on treatment.

In both acute and chronic HBV infections, we have shown the value of the additional information given by this assay in judging the stage of infection. Although the Amerlite "e" assay result is not a true quantitation, the relative value gives an indication of progression in the patient's status. The examples shown in this paper were gathered within a short time scale of use of this

assay, and we have found that the assay is continuing to provide useful additional patient information.

About 10–15% of HBsAg-positive samples may be nonreactive for HBe markers by this assay (unpublished observations); however, most of these samples are either positive for both or negative for both markers by other conventional HBeAg assays. Further follow-up samples from such nonreactive patients showed that most were in the process of seroconverting from HBeAg to anti-HBe, information that can be of value to clinician and patient.

CONCLUSION

In summary, the introduction of an enhanced chemiluminescent assay for the simultaneous testing for HBeAg and anti-HBe with numerical evaluation on a linear scale has given added value to the assay of hepatitis B e markers. The HBe assay result has given additional information about the reactivity of the sample which aids in interpretation of the results and has proved particularly useful in patient follow-up who are changing their e status naturally or through the influence of chemotherapeutic agents.

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